INHIBITORS OF REGENERATION IN TUBULARIA[1]

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When stems of *Tubularia* are removed from the colony and isolated from their hydranths, regeneration will occur in the isolated stems preferentially at the distal ends as regulated by an inherent polarity gradient (Child, 1941). Several factors, both intrinsic and extrinsic to the isolated stem, may govern and in many cases prevent regeneration. Of the parameters known to have an inhibitory effect, lowering the temperature will decrease the rate of regeneration (Moore, 1939; Moog, 1941; Berrill, 1948) but it increases the size of the reconstituting hydranths (Moog, 1941). Similarly, Torrey (1912), Miller (1937, 1939), Barth (1937, 1938, 1940), and Rose and Rose (1941) all found that a lowering of the oxygen tension will inhibit regeneration. However, certain respiratory poisons such as cyanide or urethane (Moog and Spiegelman, 1942) will inhibit regeneration without any parallel effect on respiration. Miller (1939) and Goldin (1942a) indicated that increased hydrogen ion concentration of the sea water would reverse the normal polarity of the stems or inhibit regeneration. Later, Goldin (1942b) found that at oxygen tensions favorable to regeneration, an increase of the hydrogen ion concentration by the addition of CO_2, would cause complete inhibition. It was shown by Rose and Rose (1941) that oxygen alone will not assure regeneration unless there is sufficient cut surface of the stem open to the sea water to allow release of an inhibitor, believed to be produced by tissues of the adult organism. Similar results were reported by Goldin (1942a) using explanted coenosarc fragments and Miller (1942) who varied metabolic exchange by covering portions of the perisarc. This inhibitor, presumably a metabolic substance, was collected by Rose (1940) from colony water and later (Rose and Rose, 1941) produced from an aerated, saturated mixture of cut stems and hydranths in sea water. When this water was applied to freshly amputated stems, regeneration was blocked. The active factor was rather unstable, being heat-labile but non-volatile. Hydranths alone were found to be active but there was evidence that stems also produced a substance which made them inhibitory upon one another. Later, Steinberg (1954) showed by ligaturing stems at intervals after amputation that inhibitor production within the regenerating stem begins around 30 hours post-amputation, when the distal hydranth has become well determined.

Recently, Tardent (1955) has been able to produce inhibition with "hydranth equivalent" extracts made from tissue breis of mature hydranths of *Tubularia larynx*. This substance does not lose its activity after sterilization or refrigerated storage.

While the tissue extracts and the inhibitor water have the same effect, namely general inhibition of regeneration, certain evidence suggested that the two factors

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were not identical. The present experiments were designed to localize the source and further identify the active factor in inhibitor water, and secondly to compare it with the inhibitory factor produced from tissue extracts of the adult organism.

Materials and Methods

Throughout the experiments, *Tubularia crocea* collected in the Woods Hole area was used. Since its appearance can be greatly altered according to the time of year it is collected and the prevailing seasonal conditions, often considerable difficulty is attached to its identification. As originally described by Agassiz (1862) and later by Nutting (1899) and Fraser (1944), *Tubularia* (*Parypha*) *crocea* Agassiz grows from a dense stolon mass and is separated into long pale, almost white stems from 8 to 10 cm. high. The stems are unbranched or slightly branched, annulated sparsely at intervals and the pedicel is distinctly swollen just below the base of the hydranth. The hydranths are red, with 20 to 24 proximal and the same number of distal tentacles. The gonophores (when mature) hang in long racemes between the proximal tentacles. They consist of 10 to 12 slender branches, each of which by successive branching may bear up to 8 or more medusae. The medusae are sessile, with no apparent radiating canals. At the oral end of the female medusae there are 6 to 10 crested tentacles or apical processes which are laterally compressed. The male medusae do not possess these crested structures. The coelenteron may have one or more longitudinal endodermal partitions which form two to four incomplete channels.

Additional observations have shown that the amount of branching found in this species ranges from thickly branched specimens (often caused by settling actinulae) to almost totally unbranched individuals. The mature hydranth may measure 8 to 15 mm. from tip to tip of the proximal tentacles.

Secondly, as observed by Cohen (1952) and Rose (1957) the pigmentation of the hydranth can vary from red through various intergrades of orange and yellow. In the past three summers we have noted hydranths viewed with incident light ranging from rose to orange red earlier in the summer along with various intergrades of orange, yellow or white later in the summer. The latter material tends to have long pale stems and is very sparsely branched. The color in the proboscides of the medusae usually conforms to the color of the hydranth. Another late summer variety conforming to the above description has deep red-wine colored hydranths which often exhibit medusae with a brown or golden proboscis or "core" in contrast to the hydranth color. One important difference of the late summer varieties is their resistance to higher temperature. When the sea water temperature reaches about 21° C., the former variety dies out and the warm water forms survive.

Collection of inhibitor water

Inhibitor water was obtained from stems amputated from the stolon mass with the hydranths left intact. Each stem was cut separately and transferred to fresh sea water accompanied by a minimum of debris, small organisms, etc. The hydranths were washed in filtered sea water and then transferred to an aspirator flask containing twice-filtered sea water. The number of mature hydranths varied from two to four per ml. of collecting fluid.

Air bubbles which kept the stems turning over continually were generated through the flask in one of two ways. Initially, the top of the aspirator bottle was attached to a faucet vacuum aspirator and the base of the flask fitted with a clamp-regulated tube for the air intake. Later, an ordinary aquarium aerator was attached directly to the base spout of the bottle. In both cases the bottle was inclined with the spout down and submerged in a pan of running sea water. In this manner, during operation of the pump the hydranths and stems were continually rotated and aerated. Inhibitor water was harvested after 18 to 24 hours.

The water collected was then filtered twice through No. 1 and No. 50 Whatman filter paper in a Buchner funnel, before being submitted to any other treatment. This will be referred to as plain filtered inhibitor. This solution appears slightly opaque and has a distinctive pungent odor. Microscopic examination shows that breakdown products of cellular cytolysis, bacteria and ciliate protozoans are present.

Preparation of tissue extracts

Large numbers of mature hydranths (250 to 550) were collected, washed in sterile sea water, drained and homogenized in a teflon-glass tissue homogenizer. The resultant brei was then centrifuged for 15 minutes at 1560 G. Several layers were produced. Floating at the top was a tough, dark red foam layer of intact cells, fibers and pigment, immediately followed by a short cap of fatty material. The major portion consisted of an opaque brown supernatant solution and at the bottom there was a dark red pigment layer covered by a white layer. The supernatant solution was re-centrifuged at 15,000 G for 15 minutes. The first and second sediment layers were re-suspended in filtered sea water and again centrifuged at 15,000 G for 5 minutes. The combined supernatants were re-centrifuged at 21,000 G for 15 minutes. The final supernatant was then made up to 100 ml. in filtered, bacteria-free sea water. In the final solution, each ml. of extract was equal to a known number of hydranths depending on the original number.

Throughout the following experiments, the criterion for regeneration was the degree of differentiation, *i.e.*, the number of fully differentiated hydranths per input of freshly amputated stems in standing sea water.

Stages of regeneration referred to are adopted from those described in detail by Davidson and Berrill (1948), Rose and Rose (1941) and Steinberg (1954). They are referred to as the inactive stage, pigmented band (primordia of the tentacles), proximal ridge (proximal tentacle striations), proximal-distal ridge (proximal-distal tentacle striation), pinched (constriction of the hydranth) and emerged (fully developed regenerate) stages.

Results

Before attempting to analyze the active substance in inhibitor water it was deemed necessary to determine from which tissues the inhibitor originated and which were the best sources. This necessitated finding if there was any mutual inhibitory effect of the cut stems upon each other. Both Rose and Rose (1941) and Barth (1938) pointed out that crowding freshly amputated stems would retard their regeneration.

In this experiment, a series of Stender dishes containing 18 ml. of standing sea water were filled with increasing numbers of freshly amputated stems. The

results of 6 experiments can be seen in Figure 1. All of the stems regenerated in the dishes up to 16 stems per volume and at 24 stems per dish, at least 90% of the cut stems went on to form fully differentiated hydranths at the same time as the controls, about 48 hours post-amputation. Beyond this, the number of regenerates slowly dropped off. Above an input of 24 stems, the stems which were able to regenerate, did so at a considerable time after the controls. The last two points are readings taken 70 hours after amputation. While it was remarkable that so many stems would regenerate under such crowded conditions, the rate of regeneration had clearly lagged.

These results suggested that if an inhibitor was being produced, it was occurring in sub-threshold quantities or it must come from more differentiated tissues of the regenerating hydranth. This seemed to validate an earlier report by Stein-

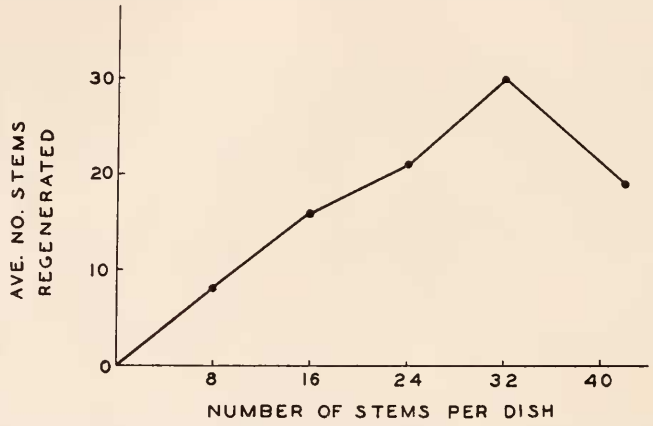

FIGURE 1. The effect of crowding upon regenerating stems in standing sea water.

berg (1954) that only later stages of differentiation produce substances which inhibit earlier stages of regenerating stems.

Living tissue explants

It had also been reported by Rose and Rose (1941) that the presence of living adult hydranths almost completely inhibited the regeneration of stems. The effect of mature hydranths alone was therefore tested on a relatively simple level of biological assay. Fixed numbers of freshly cut stems were added to standard volumes of standing sea water. To these dishes, freshly amputated hydranths were added in increasing numbers. The purpose was to find the minimum number of hydranths which would show an inhibitory effect upon the regenerating stems. This group of experiments was conducted at temperatures between 18° and 22° C.

In the first series of experiments, amputated hydranths only were placed in 18 ml. of standing sea water in Stender dishes. The number of amputated stems added was either 1, 2, 4 or 8 stems per dish as depicted by solid lines in Figure 2. Each point represents the average of six experiments. There was no inhibitory effect up to the addition of 4 hydranths per dish but as the number of freshly amputated hydranths was increased, the number of regenerates began to decrease.

Between the addition of 16 to 32 hydranths, the maximum number of regenerates was around 3 regardless of stem input. Complete inhibition of all stems occurred with the addition of 40 or more hydranths. This is in agreement with Tardent (1955) who found that tissue extracts of adult hydranths in approximately the same volume of sea water produced almost total inhibition with the addition of 40 hydranth equivalents.

When the volume of the culture medium was increased ten-fold, as represented by the dotted curve B in Figure 2, the first indication of inhibition was seen with the addition of 16 hydranths per bowl with 8 stems. Three out of 8 stems were still able to regenerate along with 64 hydranth explants.

In a second series of experiments the effects of explanted cut stems with intact hydranths upon freshly amputated stems were examined. Total prevention of re-

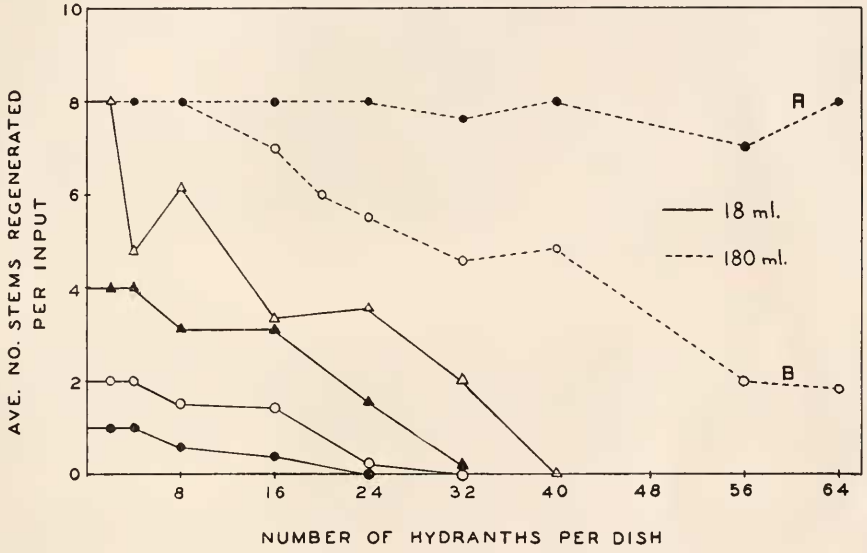

FIGURE 2. The effect of living hydranth explants upon increased numbers of regenerating stems in standing sea water. Each point represents the average of six experiments.

generation became evident when the ratio of tissue explants to regenerating stems became 4:1.

In all cases the stems which were inhibited almost always stopped their development at the stage when proximal and distal ridges were first becoming apparent. The stems which did regenerate in the presence of an increased amount of hydranth material, did so at a considerably later time than the controls. It was obvious that the rate of regeneration was retarded.

Inhibition occurred only when freshly cut stems or those in the very early stages of regeneration were tested. If the previously amputated stems had reached the stage of proximal ridge or later before being added to the culture medium, they were unaffected by the addition of hydranths. The sensitive period to the explants appears early in the regenerative phase.

These results suggested that inhibition might be caused by either an increase

in tissue mass which could result in an accumulation of metabolites, a reduction in the oxygen tension or an accumulation of CO_2. It has already been pointed out that a reduction in available oxygen and an increase in CO_2 will prevent regeneration.

Subsequently a duplicate series of dishes were set up in which the culture medium was aerated. Each dish contained 180 ml. of standing sea water and 8 stems. The dishes were aerated with an aquarium aerator and air stones. A comparison of aerated (curve A) and the non-aerated series (curve B) can be seen in Figure 2. Whereas inhibition of regenerates becomes evident in the non-aerated dishes, the effect of additional hydranths, up to the limit studied, was abrogated by aeration in all cases. The principal effect of aeration is to drive off CO_2 from the water (Emmens, 1953) and the above experiments strongly indicate that it is the factor acting here.

Since inhibitor water is usually collected in the presence of vigorous aeration, it did not seem likely that the results above were due to the same factor that is collected in inhibitor water. This conclusion was supported when identical groups of hydranths without stems were placed in standing sea water. After 24 hours, the culture solutions were harvested minus the hydranths and freshly amputated stems were added to the solution. None of the harvested solutions had any apparent inhibitor action upon the regenerating stems. It can be concluded that retardation and prevention of regeneration from both crowded stems and the addition of extra living hydranths to amputated stems are not due to the same factor which is found in inhibitor water.

Effects of inhibitor water

From a practical standpoint, the most effective source of active inhibitor water was from cut stems with the hydranths intact, obtained by the method already described. Repeated harvests of sea water obtained from cut stems only, without the hydranths, were collected under identical conditions. These solutions had no inhibitory effect when applied to freshly amputated stems.

The following sets of experiments were therefore run concurrently from repeated harvests of inhibitor water. The results from each modification of the inhibitor water are treated separately and the tabulations represent single experiments of ten amputated stems each.

Effect of plain filtered inhibitor. In each experiment, 50 ml. of newly collected inhibitor were applied to a series of freshly cut stems, 10 stems per finger bowl. The inhibitor solution used in some experiments completely inactivated most of the stems or arrested development prior to complete regeneration in the remaining stems. Other solutions caused only inactivation of some stems and retarded regeneration in other stems. Partial or total inhibition was in general correlated with the length of collecting time and the number of equivalent hydranths per ml. of collecting fluid. Occasionally a weak inhibitor solution was produced if the number of hydranths/ml. were two or less. Strong inhibitor solutions were always obtained when three or more hydranths/ml. were used.

The results in Table I-A show that when most control stems had completely differentiated or emerged, the majority of the inhibitor-treated stems remained inactive. After 50 to 70 hours post-amputation, many of the stems which had begun a retarded development reached the pinched or emerged stage. Only 9%

of the treated stems actually emerged. It can be seen further that once the treated stems were inactivated, only 7% of them recovered to begin regeneration. It was found that if these inactivated stems are removed and placed in running sea water, they will recover and go on to regenerate.

TABLE I

Comparative effects of treated inhibitor water upon regeneration of cut stems in standing sea water

Type of inhibitor preparation	Hours after amputation	Number in regenerative stages						
		Emerge	Pinch	Prox.-dist. ridge	Prox. ridge	Pigmen. band	Pre-primordia	In-active
A. Plain filtrate	28–46	5	2	1	5	27	28	132
	50–70	18	26	11		22	6	118
Control	30–52	56	41					3
	52–69	79	18					3
B. Dialysis of plain filtrate	30–46	2	2	4				52
	65–70	5	3					52
Control	45	15	3					2
Dialysis control	50	12	6	2				
C. Norite "A" adsorption of plain filtrate	40–70	28	9					2
Filtered inhibitor	40–70	7	8			5		29
Control	40–56	30						
D. Bacterial filtrate of inhibitor	32–46	5	6	6		3		70
	56–70	13	7					65
Control	37–48	69	13					7
E. Dialysis of bacteria-free inhibitor	30–46		4	3		2		81
	56–70	10	11	1		5		63
F. Dialysis of bacteria-free inhibitor in sea water plus chloromycetin	32–46	1	9	7		5		30
	56–70	14	18	3		2		23
								3
Control: Stems in plain sea water	45–56	52	8	4				6

Since Goldin (1942a, 1942b) and Miller (1942) had demonstrated that an increase in hydrogen ion concentration results in a decrease of regeneration, portions of the most potent filtered inhibitor were tested for a decrease in the pH. It was postulated that inhibition might be caused by an accumulation of CO_2 in the inhibitor water or, as both Goldin (1942a) and Miller (1942) suggested, by a

lowering of the pH due to the accumulation of acid metabolites. Several checks of the pH of plain sea water and freshly collected inhibitor water showed that there was a maximum drop of 0.57 in pH from that of normal sea water, pH 7.96. Part of the inhibition encountered might be the result of this increase in hydrogen ion tension either from CO_2 accumulation or acid-producing metabolites. The present method of collecting inhibitor water by means of vigorous aeration would tend to drive off any excess CO_2 produced in the medium which would minimize it as a source of inhibition. Goldin's figures show that a drop in initial pH of 1.12, by the addition of HCl, only reduced the number of regenerates to 7 out of 10 as compared to 9 out of 10 in the controls. He did not obtain complete inhibition with CO_2 until he had decreased the initial pH of his solutions from 1.3 to 1.8 pH units depending on the oxygen concentration. It is therefore difficult to assess the effect of increased acidity presumably brought about by acid metabolites in the present experiments.

Dialysis of filtered inhibitor. Routine preliminary tests for proteins made upon the filtered inhibitor were in general negative with the exception of a weak ninhydrin positive result. However, the latter result could be caused by various other contaminates in the inhibitor water.

The inhibitory action of plain filtered inhibitor water was therefore tested on newly cut stems after dialysis. Cellulose dialyzer tubing, 1″ flat, was thoroughly washed with running sea water, both inside and out. (This procedure was found absolutely necessary since dialyzer tubing contains a water-soluble plasticizer that is quite toxic to *Tubularia*. In fact, it was found that the rinsings of dialysis tubing would completely inhibit regeneration.) Each tube, containing 25 or 50 ml. of plain filtered inhibitor, was placed in 150 ml. of standing sea water in a finger bowl. In some cases the solution outside of the dialysis bag was a bacterial filtrate of plain sea water. Ten amputated stems were added to each bowl on the outside of the dialyzer tubing. Control dishes of amputated stems in plain bacteria-free sea water and a second control of sea water dialyzed against standing sea water containing stems were included.

The results showed that the inhibitor filtrate does dialyze and the effect of inhibition is still strongly evident after dialysis as indicated in Table I-B. When these results are compared to the inhibition produced after the direct application of the filtrate, it is seen that there was little decrease in its effectiveness even after dilution against 3 to 6 times its volume.

Treatment with Norite. Concurrent with the previous experiments, part of the filtered inhibitor was treated with Norite "A." The mixture was then filtered through Whatman No. 1 filter paper producing a clear filtrate. This solution was then applied to freshly amputated stems. The results are shown in Table I-C. It is clear that adsorption by Norite almost completely removed the effect of the inhibitor. While this treatment did eliminate the factor or factors which cause inhibition, their identity was far from established. Likewise, microscopical examination of the clear filtrate also indicated that cells in suspension and microorganisms were removed.

It was quite possible that the presence of cells or microorganisms was linked to activity of the inhibitor and experiments were devised to eliminate them. Earlier, the plain filtered inhibitor was subjected to high speed centrifugation at 21,000 G for one hour and the supernatant decanted off. Most of the bacteria would be

eliminated by this procedure. Application of this solution of cut stems showed no alteration in inhibitor activity. Since Rose (1940) had shown that heating the inhibitor to 90 or 100° C. completely inactivated it, bacterial filtration was utilized.

Action of inhibitor after bacterial filtration. A clear bacteria- and cell-free solution was obtained after passing the inhibitor solution through a Mandler bacterial filter. In each experiment, ten newly amputated stems were introduced into 50 ml. of this solution. While bacteria and other microorganisms are introduced along with the stems, their growth was never observed in the straight filtrate and the solution remained clear.

After 48 hours most of the controls had regenerated but the majority of the stems in the bacteria-free inhibitor were inactive or retarded in their development. See Table I-D. It was observed that many of the stems which began cell movement formed curious bulbular outgrowths or blebs at the distal and sometimes proximal ends of the stem. These abortive attempts to regenerate are evidence that tissue migration does occur but even the early signs of hydranth differentiation are lacking. Even so, a considerable number of retarded stems reached the fully emerged regenerative stage. It was becoming apparent that the active factor in the inhibitor was not dependent upon a continuous interaction with microorganisms. This conclusion was further supported by the next experiment.

Dialysis of bacteria-free inhibitor. In experiments which were run concurrently with those using bacteria-free inhibitor, 50 ml. of bacteria-free preparations of inhibitor were sealed in well washed dialyzer tubing. The tubing was rinsed in sterile sea water and then placed in 150 ml. of standing bacteria-free sea water in finger bowls. The inhibitive action of the bacteria-free inhibitor was still effective after dialysis as shown in Table I-E. When almost all controls had regenerated (from 45 to 56 hours), none of the treated stems had emerged. Here, again, a certain number of the retarded stems were able to recover and regenerated tardily. It can be seen by comparison with the action of filtered inhibitor alone that little activity was lost from the dilution of the inhibitor after dialysis. Since microorganisms were presumably blocked from the bacteria-free inhibitor water contained in the dialysis tubing, there does not appear to be a direct interaction between them and the inhibitor factor.

As a precaution against possible growth of microorganisms introduced on the amputated stems, part of the bacteria-free sea water was prepared with 0.002% of chloromycetin. The results shown indicated that the antibiotic offers some protection against the inhibitor. Whereas only 23% of the stems ever regenerated when treated with the bacteria-free filtrate, 53% of the stems treated with chloromycetin eventually regenerated. The greatest recovery was seen when there was partial inhibition caused by a less active inhibitor. The possible explanation for this unusual result will be discussed later.

Inhibition with adult tissue extracts

Extracts prepared from tissue breis of the entire adult hydranth were made according to the procedure stated earlier and adopted from the technique of Tardent (1955). The hydranth extracts were found highly resistant to heat of sterilization or boiling. Such treatment caused a denaturation of proteins while the remaining filtrate was still active. This filtrate could be refrigerated for several

days at 7° C. with no diminution of activity. All subsequent extracts were therefore subjected to heat sterilization and centrifuged to throw down the precipitate. Further high speed centrifugation of the extract at 21,000 G for one hour had no effect on the inhibitor activity.

Tardent's experiments were based on the addition of hydranth equivalents per 15 ml. of culture medium in which he measured the regeneration rate (length/time) of the cut stem. He produced complete inhibition after adding 20 to 40 hydranth equivalents. In experiments designed to duplicate Tardent's, we found that even the addition of one ml. of extract (equivalent to 5 hydranths) would completely

TABLE II

Effects of increasing amounts of hydranth tissue extracts on regenerating stems in standing sea water

Hours after amputation	Amount of added sterile extract. 1 ml. = 5 hydranth equiv.	Stages reached					
		Emerge	Pinch	Proximal-distal	Proximal	Pigment band	Inactive
+50	5 ml.				2	4	14 (bulbous)
	7				8		12 (bulbous)
	10						20 (bulbous)
	control	20					
+70	1 ml.	20					
	2	20					
	3	20 (stunted)					
	4	14	6				
	5		2		6	12	
	7	2	4			12	
	10						20
	control	20					
+96	1 ml.	20					
	2	20					
	3	20 (stunted)					
	4	20 "					
	5	20 "					
	7	8	2			10	
	10						20
	control	20					

stop all regeneration. This amount is much lower than that which Tardent reported necessary for complete inhibition.

In a second series of experiments shown in Table II, each bowl contained 10 stems in sea water made up to 100 ml. with increasing concentrations of extract. Each ml. of extract was equivalent to 5 hydranths. It was found that up to the addition of 3 ml. of extract, all stems could regenerate after 72 hours although they were stunted in size. The effective concentration which blocked part of the stems fell between 20 and 25 hydranth equivalents. As the concentration was increased from 5 to 7 ml. ($\frac{1}{5}$ to $\frac{1}{3}$ hydranth equivalent per ml. of culture solution) none of the treated stems had emerged after 50 hours when all the control stems had re-

generated. In spite of this, examination of the experimental stems after 96 hours showed that some of the retarded stems were capable of regeneration. Those which did regenerate did so at an extremely slow rate and always resulted in stunted individuals. In particular, the proximal and distal tentacles were smaller. Complete inhibition, with no individuals regenerating, occurred between a hydranth equivalent concentration of 35 to 50 per 100 ml. of culture fluid (a concentration of $\frac{1}{3}$ to $\frac{1}{2}$ hydranth per ml. of the culture medium).

Most of the retarded stems, particularly those in hydranth equivalent concentrations of 25 or higher, manifested large bulbous protrusions, often accompanied by concentrations of pigment at the tip but without other signs of differentiation. These were not unlike those produced by the action of inhibitor water.

Dialysis of tissue extracts. Preparations of full strength hydranth extract (25 ml.) were placed in dialysis tubing and dialyzed against 125 ml. of standing

TABLE III

Action of hydranth tissue extracts on regenerating stems after dialysis in standing sea water

Tissue extract preparation	Hours after amputation	Number regenerated
Control (stems only)	+ 50	10/10
Dialysis of plain extract	+ 36	0/10
	+ 50	0/10
Dialysis of boiled, precipitated, and centrifuged extract	+ 36	0/20
	+ 50	0/20
	+ 62	1/20
	+ 68	1/20
	+100	1/20
Dialysis of extract *after* 24 hour dialysis in running sea water	+ 50	7/10
Control—dialysis of plain sea water	+ 37	14/20
	+ 62	20/20

sea water along with 10 freshly amputated stems. Identical preparations of sterilized, precipitated and centrifuged tissue extracts were also tested. From Table III it is evident that both preparations of the hydranth extract can dialyze and inhibit regeneration of the cut stems. Dialysis tubing filled with plain sea water had no effect on the stems. Likewise, if the extract was first dialyzed against running sea water for 24 hours, the subsequent application of the dialysate to regenerating stems showed a total loss of inhibitory activity.

Preliminary identification of the inhibitor. Bacterial filtrates of inhibitor water were prepared and these submitted to general biochemical tests. Routine tests of the filtered inhibitor for protein were negative and it has been shown that the active factor in the inhibitor water is dialyzable. The filtrate consistently gave a positive test with Schiff's reagent, usually regarded as specific for aldehydes. Numerous investigators have questioned this specificity and have suggested that the reagent

will react with ketones and other substances with unsaturated hydrogen bonds but the evidence for this is quite conflicting (Hale, 1957). Other tests of the inhibitor solution for ketones were found to be negative.

Since the active factor in inhibitor water could be adsorbed on activated charcoal, preliminary investigations were made with a variety of adsorbants in a chromatographic column. A Pyrex column was employed, 24″ long × ½″ internal diameter, and fitted to a suction flask attached to a faucet vacuum. Occasionally, filtration was aided by a positive pressure head supplied from an aerator.

The inhibitor solutions were first adsorbed on Norite "A" and amberlite resins CG-45 and CG-50 and then eluted with either 2% ammonia in 50% ethanol or 1% acetic acid in sea water. The resultant elutants gave positive tests with Schiff's reagent. Application of the neutralized elutants to freshly amputated stems resulted in inhibition but these results were not conclusive due to the nature of the solvents.

As it was desired to apply these elutants to cut stems for assay, water-soluble adsorbents, magnesium oxide and aluminum oxide were used. When these columns were eluted with sea water, the elutants gave negative tests with Schiff's reagent and still retained some of the inhibitor activity. At this point it is not certain if the test substances positive to Schiff's reagent are identical with the inhibitor fraction. Further chromatographic analyses are anticipated.

Discussion

Throughout the present experiments three principal observations were associated with the inhibition of amputated stems. When the inhibitory effect fell short of causing complete inhibition, a reduction in the rate of regeneration was always noted. This was measured by the length of time necessary for the regenerate to reach the fully differentiated stage of hydranth formation. Such an effect has been reported with almost every inhibitory parameter of regeneration investigated.

Very often reduced rate was accompanied by a reduction in the size of the regenerating hydranths. Generally, size reduction may be correlated with a reduced rate but, as Moog (1941) has observed, a lowering of the temperature allows an increase in the size of the regenerate in *Tubularia*. Any regeneration rate based on length per time could therefore be subject to this and other errors. For this reason the criterion of stages in differentiation was used.

Another characteristic of inhibition was the evident prevention of differentiation even though the stems displayed activity usually associated with it. In many of the non-regenerating stems knob-like blebs of tissue were formed beyond the perisarc at the distal end. Quite often both ends of the stem were so affected. These projections were probably caused by a migration of the coenosarc since the coenosarc became visibly thinner in the center of the stem but they were never accompanied by visible differentiation. A shifting movement of the entire coenosarc toward the distal end in normal regeneration, as reflected in a gradient of optical density, has been thoroughly described by Steinberg (1954, 1955). It is likely that the cellular movement seen here is of the same nature but the prevention of regeneration in the present experiments seems to be a suppression of differentiation rather than a restriction of cell movement.

Inhibition of differentiation in an active regenerate can be produced in other

ways. Specific inhibition of differentiated parts has been strikingly demonstrated with living tissue grafts by Rose (1955, 1957). He found if grafts of developing hydranth primordia were properly orientated in a distal position to a regenerating hydranth, the grafts suppressed the differentiation of the specific like parts in the host. The influence of the graft was so strong that it could cause the regression of specific like parts already formed.

A group of experiments have been performed recently by C. Fulton (personal communication) in which he collected inhibitor water in low concentrations of streptomycin or penicillin. In most cases the growth of microorganisms was restricted and the water collected was not effective against regenerating stems. These results suggested that the inhibitor production was linked to microorganisms which are known to multiply during the collection of inhibitor water. In the present experiments precautions were taken against inclusion of microorganisms in the active portion of the inhibitor water but this does not rule out the significance of Fulton's observations, that the activity of inhibitor water might be due to by-products of bacteria.

However, there are several reasons why the inhibitor effect may not be a direct toxic agent of bacteria or other microorganisms. First, the experiments of Rose (1940), Tardent (1955) and our own show that stems alone, devoid of hydranths, do not produce inhibitor water when collected under identical conditions. Since microorganisms do grow in stem water, this water should also be inhibitory if the bacteria are producing a toxic factor. Secondly, from the present experiments it was seen that inhibition is not always a total all-or-none effect. In a weak solution of inhibitor all stems may eventually regenerate long after the controls. Occasionally 1 out of 10 treated stems will regenerate along with total regeneration in the controls. If the inhibitor were a toxic factor produced by bacteria, it would completely stop all stems from regenerating.

Other observations of Rose and Rose (1941), Steinberg (1954) and our own indicated that the inhibitor acts only during the early stages of regeneration. It is not likely that a toxic substance produced by bacteria would be so specific as to affect only one portion of the regenerative phase.

Lastly, the introduction of antibiotics to the collecting water, in addition to having a bacteriostatic action, might also greatly reduce the effectiveness of the inhibitor. As seen in the present experiments, the addition of an antibiotic, chloromycetin, somewhat suppressed the activity of the inhibitor. Antibiotics are sometimes used to remove biologically active substances from solution. Kutsky (1953, Kutsky *et al.*, 1956), working on the isolation of nucleoproteins from chick embryo extracts, used streptomycin to cause a specific precipitation of the nucleoprotein fraction from the supernatant fluid. The possibility that the same kind of action is occurring when inhibitor water is collected in the presence of antibiotics should not be overlooked.

Summary

1. A study has been made of the effects of living tissue explants, inhibitor water solutions and tissue extracts as inhibitors of regeneration in *Tubularia crocea*.

2. Stems alone have little inhibitory effect upon one another.

3. Living hydranth explants can cause complete inhibition of cut stems. The effective sensitive period or the period of developmental arrest extends from the

time of amputation to just before the proximal ridge stage. Inhibition in these cases can be cancelled by aeration and is not due to metabolic inhibitors.

4. Complete inhibition of cut stems can be produced with harvests of culture solutions taken from cut stems with intact hydranths. No inhibition was obtained with solutions taken from stems only. There is little loss in potency after filtration, centrifugation or sterilization. The active factor withstands bacterial filtration and is dialyzable. The fresh filtrate gives a positive reaction with the Schiff reagent. It is heat-labile, susceptible to cold storage and can be adsorbed on Norite "A." Part of its activity can be removed with antibiotics and it is possible to completely adsorb the inhibitor on inorganic salts and ion exchange resins.

5. The supernatants obtained from breis of hydranth tissue were also an effective inhibitor. A threshold concentration of 20 to 25 hydranth equivalents/100 ml. initiated inhibition and complete inhibition resulted from the addition of $\frac{1}{3}$ to $\frac{1}{2}$ hydranth equivalent/ml. of culture medium. Fresh or boiled tissue extract will dialyze and cause complete inhibition and prior dialysis of the extract against running sea water does alter its potency as an inhibitor. The extract is highly resistant to sterilization, centrifugation and storage.

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